Improved Throughput and Reproducibility for Targeted Protein Quantification Using a New High-Performance Triple Quadrupole Mass Spectrometer

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Overview

Purpose: Demonstrate increased sensitivity, throughput, and reproducibility for targeted protein quantification in biologically complex samples using a new triple stage quadrupole instrument and Thermo Scientific™ Pinpoint™ 1.3 software.

Methods: Thermo Scientific™ TSQ Quantiva™ triple stage quadrupole mass spectrometer equipped with a Thermo Scientific™ EASY-Spray™ source were used for all experiments. A dilution series containing enzymatic digest mixtures of BSA and Enolase spiked in *E. coli* digest matrices were used to evaluate the improved quantitative performances and reproducibility of the new instrument. A separate sample set was used to evaluate the quantitative accuracy while applying the new triple stage quadrupole instrument to more complex samples. Pinpoint 1.3 software was used for rapid selected reaction monitoring (SRM) assay development based on discovery data and data processing.

Results: The new instrument design improved SRM assay sensitivity, reproducibility, and linear dynamic range significantly. Both BSA and Enolase were detected at 1 attomol on column with excellent reproducibility. Five orders of linear dynamic range was observed.

Pinpoint software allowed rapid SRM assay development and refinement for targeted proteins. Pinpoint software also allowed automatic data processing for quantifying protein expression ratios between two different sample groups with good precision.

Introduction

The triple stage quadrupole instrument-based SRM method has emerged as the method of choice for targeted protein quantification. In order to accurately quantify proteins of interest from complex biological samples in which the concentration of targeted proteins could change over six-orders of magnitude, high sensitivity and wide dynamic range are required. In most cases, sample-specific peptide standards that can be used for SRM assay development may not be available. It is critical to have software that enables rapid SRM assay development utilizing previous discovery data.

Here we report that a new triple stage quadrupole instrument was able to push detection limits of targeted proteins down to the 1-attomol level with five orders of linear dynamic range. We also demonstrate that an optimized SRM assay to quantify 12 proteins from *E. coli* complex matrices could be developed in a few hours using Pinpoint software relying on data previously acquired in the discovery phase of the project.

Methods

Sample Preparation

Sample Set 1: A sample dilution series that spiked BSA and Enolase digest mixture into 10 ng *E. coli* digest matrix at six different concentration levels (0.0005 fmol/µl, 0.005 fmol/µl, 0.05 fmol/µl, 0.5 fmol/µl, 5 fmol/µl, 50 fmol/µl) were prepared.

Sample Set 2: Two *E. coli* digest complex samples (A and D) that contain 12 protein standards with different concentration ratios were prepared. Sample A was prepared by spiking 12 protein standard digests into 500 ng *E. coli* matrices at a static concentration level of 5 fmol/µl. Sample D was prepared by spiking 12 protein standard digests into 500 ng *E. coli* matrix at three different concentration levels of 0.5 fmol/µl, 5 fmol/µl and 50 fmol/µl.

Nano-LC:

- **System:** Thermo Scientific™ EASY-nLC™ 1000 liquid chromatograph
- **Separation Column:** EASY-Spray column (50 µm ID x 15 cm)
- **MA:** 0.1% FA-H2O
- **MB:** 0.1%FA-MeCN
- **Gradient:** 5%B – 45%B in 40 min
- **Flow rate:** 300 nL/min
- **Injection amount:** 2 µl

**FIGURE 1.** Extracted chromatograms of BSA peptide of LVNELTEFAK and Enolase peptide of AEAFVEVTK in Sample A and D, respectively. The %CVs of sample A and D are shown in Figure 6. The observed differential ratios are consistent with the expected ones. The calculated %CVs were all less than 15% (Figure 6). The calculated differential group ratios of each targeted protein between sample A and sample D are shown in Figure 6. The observed differential ratios are consistent with the expected ones. The calculated %CVs were all less than 15% (Figure 6).
**Introduction**

The triple stage quadrupole instrument-based SRM method has emerged as the method of choice for targeted protein quantification. In order to accurately quantify protein expression ratios between two different sample groups with good precision. The new instrument design improved SRM assay sensitivity, reproducibility, and data processing.

**Results**

**Quantitative Performances Acquired From the BSA-Enolase Dilution Series**

By implementing a novel ion source and optics with active ion management (AIM) technology with a 10% imbalance in the RF amplitude applied to the hyperbolic quadrupoles, the new instrument improved detection sensitivity significantly. Both BSA and Enolase peptides were clearly detected at 10 attomol levels on column in 10 ng of complex *E. coli* matrix (Figure 1). In combination with the innovative EASY-Spray nano source, the signal intensity variations from run-to-run were very small and the observed percentage of coefficient of variation was less than 7%, even at low attomol concentration levels (Figure 2). With a completely redesigned ion detection system, the dynamic range of the new instrument has also been improved significantly. Figure 3 shows that the calibration curve generated from the BSA peptide of AEFVEVTK for which five orders of linear dynamic range was observed.

**Mass Spectrometry:**

- **Instrument:** TSQ Quantiva MS with an EASY-Spray nano source
- **Spray voltage:** 1800 V
- **Sheath gas:** 0
- **Auxiliary gas:** 0
- **Capillary T:** 275 °C
- **Collision gas:** Ar, 1.5 mTorr
- **Q1 and Q3 resolution:** 0.7 Da
- **Cycle time:** 1.5 sec; CE=0.03 × m/z of precursor ion + 6.0
- **SRM:** Non-scheduled for initial SRM assay development
  - Scheduled SRM with 2 min scheduled time window for the refined SRM assay

**Data Analysis:**

Thermo Scientific™ Xcalibur™ 2.2 and Pinpoint 1.3 software were used for all data processing.

**FIGURE 1.** Extracted chromatograms of BSA peptide of LVNELTEFAK and Enolase peptide of NVNDVIAPAFVK from the dilution series (0.0005 fmol/µl)

**FIGURE 2.** The %CV for peptide LVNELTEFAK from BSA and for peptide NVNDVIAPAFVK from Enolase for the low attomol injections of the dilution series (0.0005 fmol/µl)

**FIGURE 3.** Calibration curve of spiked BSA peptide LVNELTEFAK. Over 5-orders of linear dynamic range was observed.

**FIGURE 4.** SRM development workflow using Pinpoint 1.3 software

Step 1: Refinement of SRM assay by automatically selecting up to top 5 detected fragment ions for each peptide from the discovery data. The new instrument allows running 1000 unscheduled transitions in a single HPLC run.

The calculated differential group ratios of each targeted protein between sample A and D. The observed differential ratios are in good match with the expected group ratios between sample A and sample D and the %CV of each targeted samples.

Precise relative quantification results were observed using a rapidly developed assay using the data from the initial SRM assay. Only two HPLC MS/MS runs were required to generate the final SRM assay for quantifying the 12 protein targets from both matrices for sample A and *E. coli*.

The presence of a complex matrix.

Low attomol LOD/LOQ of targeted proteins in complex biological samples was achieved with a new triple stage quadrupole instrument demonstrating ultimate quantitative performances and reproducibility for targeted protein quantification using a new high-performance triple quadrupole mass spectrometer.
Nano-LC: 0.5 fmol/concentration level of 5 fmol standards with different concentration ratios were prepared. Sample A was prepared by Sample Set 1: A sample dilution series that spiked BSA and Enolase digest mixture and by Sample Set 2: Two.

Pinpoint software relying on data previously acquired in the discovery phase of the project. We also demonstrate that an optimized SRM assay to quantify targeted proteins could change over six-orders of magnitude, high sensitivity and wide dynamic range are required. In most cases, sample-specific peptide standards that can target proteins. Pinpoint software also allowed automatic data processing for quantifying Pinpoint software allowed rapid SRM assay development and refinement for targeted proteins. Pinpoint software also allowed automatic data processing for quantifying Pinpoint software also allowed automatic data processing for quantifying.

Methods: Thermo Scientific™ TSQ Quantiva™ triple stage quadrupole mass spectrometer was used in the experiments. A dilution series containing enzymatic digest mixtures of BSA and Enolase peptides were clearly detected at 10 attomol levels on column in 10 ng of E. coli matrix at three different concentration levels of E. coli digest complex samples (A and D) that contain 12 protein matrices at a static injection amount: 2 µl, 0.05 fmol/µl, 0.5 fmol/µl, and 5 fmol/µl.

Results: The new instrument design improved SRM assay sensitivity, reproducibility, and data processing. Excellent reproducibility with %CV better than 6.5 was observed even at low attomol concentration levels. Low attomol LOD/LOQ of targeted proteins in complex biological samples was required to generate the final SRM assay for quantifying the 12 protein targets from both samples.

The optimized final SRM assay was used for both sample A and sample D. Each sample was run in triplicate. Pinpoint software was used to process all the data and calculate the group ratios between sample A and sample D and the %CV of each targeted peptides (Figure 5).

The calculated differential group ratios of each targeted protein between sample A and sample D are shown in Figure 6. The observed differential ratios are consistent with the expected ones. The calculated %CVs were all less than 15% (Figure 6).

Rapid SRM Assay Development and Refinement Using Pinpoint Software

The new triple stage quadrupole instrument was applied to determine the differential ratios of 12 proteins spiked in 500 ng of complex E. coli matrices for sample A and sample D. The SRM assay development and refinement were carried on using Pinpoint 1.3 software and previous 12 protein identification results. Figure 4 shows the steps to develop the initial SRM assay relying on the discovery data and refine the final SRM assay using the data from the initial SRM assay. Only two HPLC MS/MS runs were required to generate the final SRM assay for quantifying the 12 protein targets from both samples.

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The calculated differential group ratios of each targeted protein between sample A and sample D are shown in Figure 6. The observed differential ratios are consistent with the expected ones. The calculated %CVs were all less than 15% (Figure 6).
Nano-LC: Sample Set 1: A sample dilution series that spiked BSA and Enolase digest mixture. Here we report that a new triple stage quadrupole instrument was able to push software that enables rapid SRM assay development utilizing previous discovery data.

Dynamic range are required. In most cases, sample-specific peptide standards that can targeted proteins could change over six-orders of magnitude, high sensitivity and wide dynamic range are required. The triple stage quadrupole instrument-based SRM method has emerged as the preferred method for targeted protein quantification in biologically complex samples using a new triple stage spectrometer equipped with a Thermo Scientific™ EASY-Spray™ source were used for quantitative performances and reproducibility of the new instrument. A separate sample set was used to evaluate the quantitative accuracy while applying the new triple stage quadrupole instrument to more complex samples. Pinpoint 1.3 software was used for quantitative performances and reproducibility of the new instrument.

Overview

Purpose: Demonstrate increased sensitivity, throughput, and reproducibility for targeted protein quantification using a new triple stage quadrupole instrument which five orders of linear dynamic range was observed.

By implementing a novel ion source and optics with active ion management (AIM) which five orders of linear dynamic range was observed. Figure 3 shows that the calibration curve generated from the BSA peptide of AEFVEVTK for dynamic range of the new instrument has also been improved significantly. Figure 2 depicts the observed differential ratios of 12 proteins spiked in 500 ng of complex E. coli matrix (Figure 1). In combination with the innovative EASY-Spray nano LC-MS spectrometer equipped with a Thermo Scientific™ EASY-Spray™ source were used for mass spectrometry: Thermo Scientific™ Xcalibur™ 2.2 and Pinpoint 1.3 software were used for all data processing.

1 attomol on column with excellent reproducibility. Five orders of linear dynamic range was observed.

The new instrument design improved SRM assay sensitivity, reproducibility, and data processing.

Precise relative quantification results were observed using a rapidly developed SRM assay using the data from the initial SRM assay. Only two HPLC MS/MS runs were required to generate the final SRM assay for quantifying the 12 protein targets from both sample A and D. The observed differential ratios are in good match with the expected ones. The calculated %CVs were all less than 15% (Figure 6).

Step 1: Develop initial SRM assay by selecting all identified peptides from the previous discovery experiments.

Step 2: Generating the initial SRM assay by selecting top six detected y-type fragment ions for each peptide from the discovery data. The new instrument allows 500 SRM transitions per second, providing rapid initial SRM assay development by running 1000 unscheduled transitions in a single HPLC run.

Step 3: Refinement of SRM assay by automatically selecting up to top 5 detected peptides per protein from the initial run. All transitions are scheduled automatically for the refined SRM assay.
Nano-LC: concentration level of 5 fmol/spiking 12 protein standard digests into 500 ng Sample Preparation

E. coli 12 proteins from

Here we report that a new triple stage quadrupole instrument was able to push software that enables rapid SRM assay development utilizing previous discovery data. Targeted proteins could change over six-orders of magnitude, high sensitivity and wide proteins of interest from complex biological samples in which the concentration of The triple stage quadrupole instrument-based SRM method has emerged as the protein expression ratios between two different sample groups with good precision. Pinpoint software also allowed automatic data processing for quantifying Pinpoint software allowed rapid SRM assay development and refinement for targeted proteins. Pinpoint 1.3 software was used for quantitative performances acquired from the BSA-Enolase dilution series.

Results:

Thermo Scientific™ TSQ Quantiva™ triple stage quadrupole mass spectrometer equipped with a Thermo Scientific™ EASY-Spray™ source were used for

Methods:

Thermo Scientific™ Xcalibur™ 2.2 and Pinpoint 1.3 software were used for all data analysis. Data analysis:

Thermo Fisher Scientific, San Jose, CA

Overview

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Conclusion

- Low attomol LOD/LOQ of targeted proteins in complex biological samples was achieved with a new triple stage quadrupole instrument demonstrating ultimate sensitivity for peptide quantification.
- Excellent reproducibility with %CV better than 6.5 was observed even at low attomol concentration levels.
- In excess of five-orders of linear dynamic range was observed for a peptide in the presence of a complex matrix.
- In combination of Pinpoint 1.3 software and fast SRM scan speed of the new triple stage quadrupole instrument, an optimized SRM assay to quantify 12 proteins in complex E. coli matrix was developed in a few hours requiring only two iterative experiments.
- Precise relative quantification results were observed using a rapidly developed SRM assay in four iterations of optimization using Pinpoint software.

Discussion:

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- Precise relative quantification results were observed using a rapidly developed SRM assay in four iterations of optimization using Pinpoint software.

Figure 1: Extracted chromatograms of BSA peptide of LVNELTEFAK and

Figure 2: Observed differential ratio of each targeted protein between samples A and D. The observed differential ratios are in good match with the expected ratios.

<table>
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<tr>
<th>Protein Target</th>
<th>Expected Ratio (sample A/D)</th>
<th>Observed Ratio (sample A/D)</th>
</tr>
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<tbody>
<tr>
<td>Human Acid Glycoprotein</td>
<td>0.10</td>
<td>0.12</td>
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<tr>
<td>Alcohol Dehydrogenase</td>
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<td>Human Alpha Lactalbumin</td>
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